| FORM PTO-1390 (Modified) (REV 11-98) | U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE **TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371** | ATTORNEY'S DOCKET NUMBER 41934/23838 |
|---|---|---|
| | | U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 09/673785 |

| INTERNATIONAL APPLICATION NO. PCT/GB99/01211 | INTERNATIONAL FILING DATE 21.04.99 | PRIORITY DATE CLAIMED 22.04.98 |
|---|---|---|

**TITLE OF INVENTION**
PEPTIDE FRAGMENTS OF MURINE EPIDERMAL GROWTH FACTOR AS LAMININ RECEPTOR TARGETS

**APPLICANT(S) FOR DO/EO/US**
**THE QUEEN'S UNIVERSITY OF BELFAST**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))

    a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).

    b. ☐ has been transmitted by the International Bureau.

    c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☒ A copy of the International Search Report (PCT/ISA/210).

8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))

    a. ☒ are transmitted herewith (required only if not transmitted by the International Bureau).

    b. ☐ have been transmitted by the International Bureau.

    c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.

    d. ☐ have not been made and will not be made.

9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).

11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).

12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

**Items 13 to 20 below concern document(s) or information included:**

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

15. ☐ A **FIRST** preliminary amendment.

16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.

17. ☐ A substitute specification.

18. ☐ A change of power of attorney and/or address letter.

19. ☒ Certificate of Mailing by Express Mail

20. ☐ Other items or information:

PCTUS1/REV03

| U.S. APPLICATION NO. (IF KNOWN-SEE 37 CFR 09/673785 | INTERNATIONAL APPLICATION NO. PCT/GB99/01211 | ATTORNEY'S DOCKET NUMBER 41934/23838 |
|---|---|---|

21.    The following fees are submitted:.

**CALCULATIONS   PTO USE ONLY**

**BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5)) :**

☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO . . . . . . . . . . . .   $1,000.00

☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO . . . . . . . . .   $860.00

☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO . . . . . . . . . . . .   $710.00

☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) . . . . . . . . . . . .   $690.00

☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . . . . . . .   $100.00

| ENTER APPROPRIATE BASIC FEE AMOUNT = | $860.00 | |
|---|---|---|

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20  ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

| | | | | $0.00 | |
|---|---|---|---|---|---|
| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | | |
| Total claims | 8    - 20 = | 0 | x   $18.00 | $0.00 | |
| Independent claims | 3    - 3 = | 0 | x   $80.00 | $0.00 | |
| Multiple Dependent Claims (check if applicable). | | | ☒ | $270.00 | |

| TOTAL OF ABOVE CALCULATIONS   = | $1,130.00 | |
|---|---|---|

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable).**   ☐   $0.00

| SUBTOTAL  = | $1,130.00 | |
|---|---|---|

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20  ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).   +   $0.00

| TOTAL NATIONAL FEE   = | $1,130.00 | |
|---|---|---|

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**   ☐   $0.00

| TOTAL FEES ENCLOSED   = | $1,130.00 | |
|---|---|---|
| | Amount to be: refunded | $ |
| | charged | $ |

☐   A check in the amount of                    to cover the above fees is enclosed.

☒   Please charge my Deposit Account No.      **20-0823**      in the amount of  **$1,130.00**      to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒   The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment
to Deposit Account No.      **20-0823**      A duplicate copy of this sheet is enclosed.

**NOTE:  Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

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**20 OCTOBER 2000**
DATE

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422 Rec'd PCT/PTO  2 0 OCT 2000

# PEPTIDE FRAGMENTS OF MURINE EPIDERMAL GROWTH FACTOR AS
## LAMININ RECEPTOR TARGETS

1

2

3      This invention relates to the use of (synthetic and

4      modified) laminin receptor-targetted ligands for the

5      treatment of angiogenic diseases such as proliferative

6      retinopathies and metastatic cancer as well as for the

7      treatment of Candida spp. infections, or parastic

8      infestations such as leishmania and trichomonas

9      vaginalis.

10

11     Laminin antagonists (which are anti-angiogenic) can be

12     used to inhibit secondary tumour spread (by inhibiting

13     tumour cell attachment) and to prevent growth of

14     metastatic secondaries (by inhibiting

15     neovascularisation).  These antagonists could also be

16     used to treat other angiogenic disorders (such as

17     diabetic retinopathy).

18

19     Laminin agonists (which promote angiogenesis) could be

20     used to treat retinopathy of prematurity, and could

21     also be used to promote wound healing (for example in

22     corneal epithelium).

23

24     Both the antagonists and the agonists would be expected

25     to inhibit parasite binding to tissue surfaces and

26     would thus prevent infection or infestation.

27

28     Angiogenic diseases are those disorders which are

29     directly caused by, or complicated by the inappropriate

30     growth of new blood vessels.  The major angiogenic

31     diseases include the common metastatic solid tissue

1  cancers (breast, gastrointestinal, lung, prostatic,
2  etc), diabetic retinopathy, neovascular glaucoma,
3  rheumatoid arthritis and psoriasis. Angiogenesis is
4  the rate-limiting step in the growth of secondary
5  tumours; inhibition of their neovascularisation is
6  known to stop their growth.
7
8  In this field it is already known that the native
9  ligand of the 67kDa laminin receptor (67LR) is
10  encompassed by the linear sequence of amino acids 925-
11  933 of the laminin $\beta$-1 (previously known as laminin B1
12  or b1) chain (numbering refers to the mature murine
13  laminin $\beta$-1). Synthetic laminin $\beta$-1$_{925-933}$ (single letter
14  amino acid code: CDPGYIGSR-NH$_2$) has been shown to
15  inhibit tumour establishment in mice, by inhibiting
16  attachment of tumour cells to basement membranes. It
17  has also been demonstrated that laminin $\beta$-1$_{925-933}$
18  inhibits angiogenesis in the chick.
19
20  However, synthetic laminin-derived peptide (laminin
21  $\beta$-1$_{925-933}$) stimulates angiogenic events in mammalian
22  cells (in which it acts as a pure 67LR agonist), making
23  it useless as the basis of a human therapy.
24
25  It is one object of the present invention to provide a
26  medicament to treat angiogenic diseases.
27
28  The present invention provides a peptide factor derived
29  from murine epidermal growth factor (EGF) peptide for
30  use in the preparation of a medicament for the
31  treatment of angiogenic diseases.
32
33  The mechanism by which EGF derived peptides inhibit new
34  blood vessel formation is through their antagonism of
35  the high affinity 67 kDa laminin receptor (67LR) found
36  on endothelial cells.

1    The peptides have the additional effect of inhibiting
2    tumour cell attachment to basement membranes, and may
3    be used to prevent solid cancer spread in cases where
4    cancer cells have been identified circulating in the
5    blood.
6
7    Modified peptides may be protected from proteolytic
8    degradation by substitution of key residues with
9    unnatural amino acid analogues at susceptible bonds,
10   such as tyrosine analogues (at position 5) and arginine
11   analogues (at position 9). The peptides may be capped
12   at *N*- and *C*-termini (with acetyl and amide groups
13   respectively) and at the thiol groups of the cysteines
14   (with acetamido methyl groups).
15
16   Typically the peptide is an antagonist of the 67kDa
17   Laminin Receptor (67LR).
18
19   The peptide factor is based on amino acid residues 33
20   to 42 of murine epidermal growth factor (mEGF).
21
22   The amino acid sequence of mEGF- (33-42) is CVIGYSGDRC.
23
24   Preferably the sequence of peptide factor is modified
25   from the natural sequence to protect the peptides from
26   protease attack.
27
28   Preferred substitutions include the use of tyrosine
29   analogues at position 5 and arginine analogues at
30   position 9.
31
32   Preferably the peptide factor is capped at the N
33   terminal with an acetyl group.
34
35   Preferably the peptide factor is capped at the C
36   terminal with an amide group.

4

1    Preferably the thiol groups of cysteines are capped

2    with acetamido methyl groups.

3

4    In one embodiment the synthetic peptide has the

5    sequence

6

7        Acetyl-C-[S-Acm]-VIGYSGDR-C-[S-Acm]NH$_2$
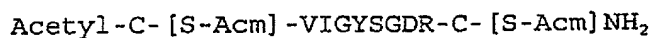
8

9    A preferred tyrosine analogue is Tic-OH.

10

11   A preferred arginine analogue is Citrulline.

12

13   The structure of Citrulline and other potential

14   arginine analogues are shown below.
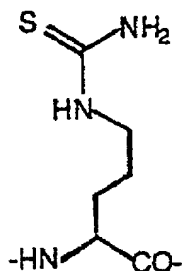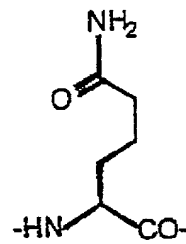
15

16                **Citrulline and analogues**

17

18

19

20

21

22

23

24

25
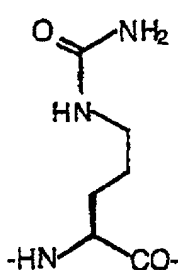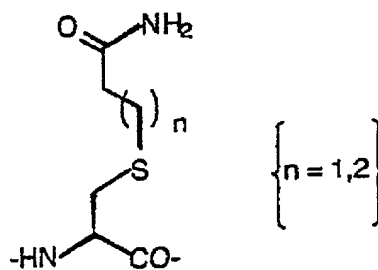
26         citrulline        cysteine-derived analogues

27                  {prepared by reaction of cysteine with

28                  Br-(CH$_2$)$_n$-CONH$_2$}

29

30

31

32

33

34

35

36        thiono-citrulline        homo-glutamine

{prepared by reaction of ornithine with ammonium
isothiocyanate}

5

1    Preferably the peptide is truncated to a shorter

2    peptide without losing its antagonistic character.

3

4    The invention further provides a peptide agonist.

5

6    The agonist may be the native sequence (single letter

7    amino acid code:CDPGYIGSR-$NH_2$) or may have the tyrosine

8    substituted by any of a variety of tyrosine analogues

9    such as the comformationally restricted Tic-OH or

10   2',6'-dimethyl-beta-methyl-tyrosines, 2-O-methyl and 2-

11   O-ethyl-tyrosine and the like.

12

13   The agonist may be useful in healing endothelial cell

14   wounding.

15

16   For example, corneal endothelial cells can be damaged

17   during cataract operations and this damage does not

18   self-repair because these endothelial cells do not

19   divide.  Healing can only be effected by cell migration

20   and spreading, and this may be promoted by the agonist.

21

22   In order to explore possible conformations for the

23   parent $mEGF_{33-42}$ peptide, it was modelled using molecular

24   dynamics.  Based on these conformations a strategy has

25   been predicted to provide proteolytic protection by

26   being able to identify residues that are important to

27   the maintenance of a three-dimensional conformation

28   essential for 67LR recognition.

29

30   The following is a description of some examples of

31   modifications and uses of the invention.

32

33   1.   On the basis of the modelled structures, it was

34        found that the arginine residue participated in H-

35        bonding, and speculated that this charge may not

36        be important.  A peptide was synthesised based on

6

1    mEGF$_{33-42}$, in which the arginine residue at position
2    41 was replaced by citrulline (an uncharged
3    arginine mimetic with similar H-bonding
4    potential). This peptide provided to act as a
5    more potent 67LR antagonist and was found to be
6    resistant to trypsin degradation.
7
8    2.   Double substitution of tyrosine$_{37}$ with Tic-OH and
9         arginine$_{41}$ with citrulline, to produce a mEGF$_{33-42}$-
10        derived peptide resistant to both chymotrypsin-
11        like and trypsin-like proteases.
12
13   3.   Replacement of susceptible peptide bonds in mEGF$_{33-}$
14        $_{42}$ with protease-resistant peptide bond isosteres
15        (such as thionopeptide or methylene amino bonds).
16
17   4.   Conformationally restricted analogues may give
18        improved potency due to the essential 3-
19        dimensional conformation being stabilised. For
20        example, it should be possible to increase the
21        rigidity of the molecule by replacing each of the
22        central glycine residues in turn by $\alpha,\alpha$-dialkyl
23        substituted amino acids such as $\alpha$-amino isobutyric
24        acid (AIB) or aminocyclopropane carboxylic acid
25        (ACPCA). Alternatively, the helical turn (which
26        we have identified as essential) could be
27        stabilised by bridging with suitable intra-chain
28        linkers, such as a disulphide bond between *N*- and
29        *C*-terminal [*D*] or [*L*]-cysteines.
30
31   **EXAMPLE 1**
32
33   The invention is demonstrated with reference to
34   the following figures wherein.
35
36   Figure 1a depicts a flat mount retina showing the

7

1    effects of ROP and Figure 1b depicts a retina from
2    laminin-agonist treated mouse showing re-
3    canalisation of vessels.
4
5
6    **Treatment of Retinopathy of Prematurity (ROP)**
7
8    Severely premature babies are at risk of
9    developing retinopathies due to their being
10   exposed to high oxygen levels post-partum.  This
11   life-saving intervention compensates for poor lung
12   development but has the unfortunate side-effect of
13   causing unnaturally hyperoxic conditions in the
14   retina.  The direct effect of this is to remove
15   the normal hypoxic cues for endothelial migration,
16   resulting in inhibition of capillary growth and
17   vaso-obliteration.  When these babies are returned
18   to room air, hypoxic stimuli are restored and
19   retinal angiogenesis is again induced.  However,
20   the newly induced angiogenesis is chaotic and
21   uncontrolled, often resulting in abnormal
22   penetration of vessels into the vitreous (see
23   Figure 1a, below).  It is the uncontrolled growth
24   of these blood vessels that ultimately leads to
25   loss of visual activity.
26
27   It has now been shown that laminin agonist
28   treatment can reverse the effects of both hyper-
29   oxic induced vaso-ablation as well as norm-oxic-
30   induced angiogenesis in a murine model of
31   retinopathy of prematurity (ROP).  In this model,
32   development of ROP can be prevented by treatment
33   of neonates with daily injections
34   (intraperitoneal) of $10\mu g$ of synthetic laminin $\beta$-
35   $1_{925\text{-}933}$ (also referred to as laminin $B1_{925\text{-}933}$, single
36   letter amino acid code:CDPGYIGSR-NH$_2$).  See Figure

8

| | |
|---|---|
| 1 | 1b in comparison with 1a. Treatment with laminin |
| 2 | agonist (Figure 1b) prevents the uncontrolled |
| 3 | angiogenic response of ROP (Figure 1a) and |
| 4 | promotes re-canalisation of areas of vaso- |
| 5 | obliteration. |
| 6 | |
| 7 | The invention is demonstrated with reference to |
| 8 | the following figures wherein Figure 1a depicts a |
| 9 | flat mount retina showing the effects of ROP |
| 10 | |
| 11 | Figure 1b depicts a retina from laminin-agonist |
| 12 | treated mouse showing re-canalisation of vessels. |
| 13 | |
| 14 | Murine model of proliferative retinopathy |
| 15 | |
| 16 | Litters of 7 day old C57-BL/6J mice, together with |
| 17 | their nursing dams, are exposed to 80% oxygen in |
| 18 | an incubator maintained at 23°C and with a gas |
| 19 | exchange of 1.5L/min for 5 days according to the |
| 20 | protocol described by Stitt et al. (1998). On |
| 21 | postnatal day 12 (P12) the animals are returned to |
| 22 | room air and sacrificed at various times post- |
| 23 | hyperoxia. Animals are treated with daily i.p. |
| 24 | injections of either laminin agonists (10µg per |
| 25 | head per day) or vehicle control. Groups of room |
| 26 | air controls are maintained in parallel with |
| 27 | hyperoxia-exposed animals. Home Office project |
| 28 | and personal licenses are held for this work. All |
| 29 | animals are housed and maintained in accordance |
| 30 | with the ARVO regulations for animal care in |
| 31 | research. |
| 32 | |
| 33 | Animals are sacrificed at pre-determined key |
| 34 | stages in the vaso-obliteration (P7-P12), |
| 35 | ischaemia (P12 onwards) and vaso-proliferative |
| 36 | responses (P12-21). At sacrifice, terminally |

9

1   anaesthetised animals have a single eye enucleated

2   and the retina removed to be snap-frozen for later

3   RNA-extraction (see below).  The fellow eye is

4   either perfused with fluorescein dextran or

5   enucleated and fixed in 4% paraformaldehyde for

6   histology, immunohistochemistry and *in situ*

7   hybridisation.

8

9   <u>ALTERNATIVE USES</u>

10

11  1.   **Treatment of corneal wounds**

12

13  The cornea is a delicate transparent structure.

14  Being avascular, corneal wound healing depends

15  upon local self-renewal of the corneal epithelium.

16  This, in turn, depends upon the presence of a

17  mitogenically functional stem cell population

18  ('limbal cells'), which produce replacement cells

19  that migrate and desquamate at the denuded area.

20  Damage to these underlying stem cell populations

21  causes inappropriate re-epithelialization by

22  conjunctival cells followed by matrix deposition

23  and scar formation.  The damaging agent may be

24  corrosive chemical or heat burns, erosion by

25  contact lenses, Stevens Johnson disease.

26

27  It is known that transplantation of limbal cell

28  autografts from the unaffected eye can restore a

29  stable healing of the corneal epithelium (Kenyon

30  et al., 1996).  It has been proposed that

31  harvesting small samples of limbal stem cells,

32  followed by serial culture in vitro would provide

33  greater chance of success (particularly when both

34  eyes are affected) De Luca, et al., 1997).

35  However, with both protocols, correct uptake and

36  controlled migration of these grafted cells into

10

1       the corneal epithelium has not been optimised.

2

3       We propose that laminin agonists could be used to

4       stimulate the migratory response of the cells

5       prior to grafting, or alternatively topical

6       application of laminin agonists to the wound site

7       could be used to direct migration of the grafted

8       cells to the correct (denuded) area of the cornea.

9

10   2.    Some microbial pathogens such as *Candida albicans*,

11         express 67LR and use this as a means of attaching

12         to human basement membranes.  It is conceivable

13         that such infections could be abolished by

14         treatment with $mEGF_{33-42}$-derived peptides, which

15         would prevent the microbes from adhering to the

16         host.

17

18   **EXAMPLE 2**

19

20   **Peptide Study**

21

22   The purpose of the investigation was to determine the

23   molecular target of $mEGF_{(33-42)}$ and to identify the amino

24   acids that are essential for receptor recognition. In

25   addition, the key residues which confer laminin

26   antagonism on $mEGF_{(33-42)}$ were examined.

27

28   Two lead compounds were investigated; synthetic laminin

29   $\beta$-1 sequence $CDPGYIGSR-NH_2$ and $mEGF_{(33-42)}$ sequence

30   $AcC(Acm)-VIGYSGDRC-(Acm)-NH_2$. Bearing in mind the pure

31   antagonism of the murine EGF peptide, the aims of this

32   study were to identify the key residues responsible for

33   these contrasting activities using alanine scanning, in

34   the context of developing anti-angiogenic drugs for

35   retinopathy treatment.

36

11

1   In addition, using residue exchange between the two

2   peptides and molecular modelling to predict three-

3   dimensional structure, we wished to further investigate

4   the role of individual $mEGF_{(33-42)}$ residues in laminin

5   antagonism. A logical series of peptides was

6   synthesised and screened for receptor interaction, cell

7   adhesion and motility properties (Table 1a and 1b).

8

9   MATERIALS AND METHODS

10

11  **Peptide synthesis**

12

13  Peptide sequences based on and $mEGF_{(33-42)}$ were

14  synthesised on a model 432A peptide synthesizer

15  (Applied Biosystems, Warrington, UK), using standard

16  solid-phase Fmoc procedure (Fields 1990). Synthesis of

17  the peptides required successive additions of

18  derivatized amino acids to form a linear product.

19

20  Peptides were purified after synthesis using reverse

21  phase HPLC and purity confirmed by automated amino acid

22  analysis and electrospray mass spectrometry. All

23  peptide sequences were stored in the presence of

24  desiccant at -20°C until required for biological assay.

25

26  **Laminin receptor antibody production**

27

28  **a. Preparation of MAPs**

29

30  The peptide sequence (PTEDWSAQPATEDWSAAPTA),

31  corresponding to the COOH-terminal end of the human

32  laminin receptor, was used as the antigen template.

33  Derivation of the peptide, based on a CN-Br cleavage

34  fragment of the cDNA sequence encoding human laminin

35  receptor, has been described elsewhere (Wewer et al

36  1986). The antigen was synthesised as an octomeric

12

1    peptide derivative (MAPs) using automated Fmoc

2    procedure (Tam 1988).

3

4

5    Table 1a: Peptide substitution

| mEGF(33-42) | acetyl | ACM Cys | Val | Ile | Gly | Tyr | Ser | Gly | Asp | Arg | ACM Cys-NH₂ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I | acetyl | ACM Cys | Val | Ile | Gly | Tyr | **Ile** | Gly | Asp | Arg | ACM Cys-NH2 |
| II | acetyl | ACM Cys | Val | Ile | Gly | Tyr | Ser | Gly | **Ser** | Arg | ACM Cys-NH₂ |
| III | acetyl | ACM Cys | Val | Ile | Gly | Tyr | **Ile** | Gly | **Ser** | Arg | ACM Cys-NH₂ |
| IV | acetyl | ACM Cys | Val | Ile | Gly | Tyr | Ser | Gly | Asp | **Cit** | ACM Cys-NH₂ |
| V | acetyl | ACM Cys | Val | Ile | Gly | **OH Tic** | Ser | Gly | Asp | Arg | ACM Cys-NH₂ |

Table 1b: Peptide substitution (alanine scanning)

| mEGF(33-42) | acetyl | ACM Cys | Val | Ile | Gly | Tyr | Ser | Gly | Asp | Arg | ACM Cys-NH₂ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| VI | acetyl | ACM Cys | Val | **Ala** | Gly | Tyr | Ser | Gly | Asp | Arg | ACM Cys-NH₂ |
| VII | acetyl | ACM Cys | **Ala** | Ile | Gly | Tyr | Ser | Gly | Asp | Arg | ACM Cys-NH₂ |
| VIII | acetyl | **Ala** | Val | Ile | Gly | Tyr | Ser | Gly | Asp | Arg | ACM Cys-NH₂ |
| IX | acetyl | ACM Cys | Val | Ile | Gly | Tyr | Ser | Gly | Asp | Arg | **Ala-NH₂** |
| X | acetyl | ACM Cys | Val | Ile | Gly | Tyr | **Ala** | Gly | Asp | Arg | ACM Cys-NH2 |

13

1  b. Immunisation schedule

2

3  A pre-immune test bleed (5ml) was obtained from the

4  marginal ear vein of a male New Zealand White rabbit

5  (3.2 kg). The bleed was allowed to clot for 2 h at room

6  temperature after which its edge was detached from the

7  wall of the collection vessel. The clot was then

8  allowed to contract overnight at 4°C. Serum was then

9  removed and the residual material pelleted out by

10  centrifugation (10 min at 2,500 g). Extracted serum

11  (3.5 ml) was then frozen at -20°C until required.

12

13  Immunogen was prepared by the emulsion of MAPs (0.5 g

14  antigen in 0.5 ml PBS) in an equivalent volume of

15  adjuvant (Alum Imject; Pierce, Chester, UK). The

16  animals immune system was primed by introducing

17  immunogen (50 $\mu$g) through subcutaneous injection at

18  different sites on the animals back. The rabbit was

19  boosted by both subcutaneous and intramuscular

20  injection, 21 days after priming, using an increased

21  dose of immunogen (800 $\mu$g). Subsequent boosts were

22  performed by intramuscular injection after a further 14

23  days (800 $\mu$g immunogen), and thereafter at 21 day

24  intervals.Test bleeds were taken 2 days after each

25  boost and the serum extracted as described above. The

26  animal was boosted and bled a total of three times.

27

28  c. Enzyme-linked immunoabsorbent assay

29

30  ELISA was used to determine the specificity of the

31  antibody prepared against the synthetic MAPs peptide

32  and to determine the efficacy of binding with respect

33  to that of the linear precursor.

34

35  Peptides were dissolved in distilled water and diluted

1    to 10 μg/ml in coating buffer. Aliquots (100 μl) of
2    either linear or MAPs peptide were then added to the
3    wells of microtitre plates (Microtest III; Becton
4    Dickinson Ltd., Oxford, UK) and incubated overnight at
5    37°C. The wells were then rinsed with 100 μl wash
6    buffer and air dried. Excess adsorption sites were
7    blocked (1 h incubation at 22°C) by the addition of 10%
8    casein in PBS (0.1 ml/well). Subsequent to the removal
9    of casein solution by aspiration, wells were again
10   rinsed with wash buffer and air dried.
11
12   Antisera or pre-immune sera were then serially diluted
13   in PBS and 100 μl of each incubated in peptide coated
14   wells for 1 h at 37°C. After rinsing (0.1 ml wash
15   buffer), 100 μl per well of 5 μg/ml secondary antibody
16   (horse-radish peroxidase-conjugated goat anti-rabbit
17   IgG; Amersham International, Aylesbury, UK) was added
18   to each well and the plates incubated at 37°C for 1 h.
19
20   Wells were again rinsed with wash buffer and 0.1 ml
21   substrate solution (TMB peroxidase) added to each. The
22   plate was then incubated at 22°C for 30 min and the
23   colour reaction stopped by the addition of 0.5M $H_2SO_4$
24   (0.1 ml/well). Absorbence was measured at 450 nm on a
25   Titertek Multiscan plate reader.
26
27   d. Purification of IgG fraction
28
29   Anti-laminin receptor antiserum was purified using
30   immobilised protein G-sepharose columns (Pharmacia
31   Biotech, Uppasla, Sweden). The columns were
32   equilibrated with 20 ml sodium phosphate buffer (pH
33   7.0). Antiserum was diluted 1:4 in the same buffer and
34   a 1 ml aliquot loaded onto the column (flow rate 150
35   ml/h, fraction size 2.5 ml). After exclusion of the

15

1  unbound fraction, as determined by absorbence at 280nm,

2  the IgG component of the antiserum was eluted with 0.1M

3  glycine-HCl (pH 2.7), into tubes containing 0.1 ml Tris

4  (1M), pH 9.0. The eluted IgG fractions were bulked and

5  stored at -20°C until required.

6

7  **Maintenance of cell cultures**

8

9  Cancer and endothelial cells were maintained in either

10 DMEM (T47-D) or RPMI (SK HEP-1) media, supplemented

11 with 10% FCS, 100 IU/ml penicillin and 100 $\mu$g/ml

12 streptomycin. Cells were incubated at 37°C in a

13 humidified atmosphere of 95% air: 5% $CO_2$ and media

14 refreshed as required. Cultures (at 80-85% confluence)

15 were routinely passed on removal from monolayer by the

16 action of trypsin (0.25%) and EDTA (0.02%) in CFS.

17

18 The viability of cell populations following

19 trypsinisation was determined by the trypan blue vital

20 dye exclusion test. Populations confirmed as being in

21 excess of 95% viable were used in all studies.

22

23 Media were screened for possible bacterial or fungal

24 contamination by incubating 1ml aliquots with both

25 nutrient and Saboraud dextrose broths (Oxoid Ltd.,

26 Basingstoke, UK). Cell populations were routinely

27 monitored for sub-clinical infections by periodically

28 culturing in the absence of antibiotics.

29

30 Both cell lines and media were examined for the

31 presence of contaminating *Mycoplasma* spp. by the method

32 of Chen (1977).

33 **Determination of cell numbers**

34

35 Single cell suspensions were quantified using an

16

1   automated counter (Coulter Electronics, Harpenden, UK).
2   A 1 ml aliquot of cell suspension was diluted 1 in 20
3   in Isoton and 0.5 ml samples counted. The mean of 5
4   counts was taken and the total number of cells
5   determined. Estimates of cell number were confirmed by
6   counting in a haemocytometer.

7

8   For microtitre end-point assays, cell numbers were
9   estimated from the crystal violet staining index of the
10  cell line (Kanamaru and Yoshida 1989). Briefly, after
11  removal of media from the assay system cells were fixed
12  with formaldehyde (10% in PBS), and washed with
13  distilled $H_2O$. Aliquots (100 $\mu$l) of crystal violet
14  solution (0.1% in distilled $H_2O$) were added to each well
15  and the plates allowed to stand for 30 min. Excess
16  stain was removed by rinsing with distilled $H_2O$ (3 x 100
17  $\mu$l). The wells were then air-dried and the remaining
18  crystal violet extracted with 100 $\mu$l acidified
19  methanol. Absorbance at 620 nm was determined using a
20  Titertek Multiscan spectrophotometer.

21

22  **Proliferation assays**

23

24  The effects of synthetic peptides and growth factors on
25  the growth of breast cancer and endothelial cells were
26  determined as detailed.

27

28  Exponentially growing cells were harvested by
29  trypsinisation, as previously described. After rinsing
30  and resuspending in the relevant culture media
31  (containing 10% FCS), the cells (100 $\mu$l aliquots) were
32  dispensed into 96-well microtitre plates at a
33  population density of 2 x $10^4$ cells/well (6 wells per
34  experimental condition). Cells were the incubated for
35  24 h at 37°C after which the media was removed and the

17

1  wells rinsed with CFS (3 x 100 $\mu$l), to rid the plates
2  of cells in suspension. Media was then replaced with
3  that containing the relevant controls or treatment
4  supplements as detailed in individual experiments.
5
6  Cell numbers were evaluated spectrophotometrically at
7  620 nm, over the period of assay, after fixing with 10%
8  formaldehyde and staining with crystal violet.
9
10  Proliferative responses were analysed using the
11  Wilcoxan Rank test and significant differences at the $p$
12  < 0.05 level, defined. Results of all growth studies
13  were confirmed in at least 3 individual experiments.
14
15  **Laminin attachment assay**
16
17  Non-tissue culture grade 96-well plates, coated with
18  2.5 $\mu$g murine laminin in 50 $\mu$l CFS per well, were air-
19  dried overnight at room temperature. Preliminary
20  experiments indicated that cell attachment was
21  concentration dependent; maximal binding occurred at a
22  laminin coating of 2.5 $\mu$g/well. After rinsing with CFS
23  (100 $\mu$l), the plastic was saturated with casein (0.2%
24  in CFS). Plates were incubated at room temperature for
25  45 min then washed extensively with CFS (3 x 100 $\mu$l).
26
27  After removal of culture media, cells were detached
28  from monolayers by the action of EGTA (0.02% in CFS) at
29  37°C. The cells were then centrifuged at 800 g for 2
30  min and the pellet resuspended in DMEM (T-47D) or RPMI
31  (SK HEP-1).
32
33  Cells, at a population density of $10^6$ cells/ml, were
34  then aliquoted (1 ml) into microfuge tubes containing
35  the individual peptide sequences and incubated for 1 h

18

1    at 37°C. The cells (100 $\mu$l aliquots) were then added to

2    the pre-coated multi-well plates and incubated for a

3    further 60 min. Incubation media were removed and the

4    wells washed with CFS (3 x 100 $\mu$l) to rid the plates of

5    non-adherent cells.

6

7    Attached cell numbers were evaluated

8    spectrophotometrically at 620 nm after fixing with 10%

9    formaldehyde and staining with crystal violet.

10

11   **Attachment to mEGF$_{(33-42)}$**

12

13   That mEGF$_{(33-42)}$ bound to the 67kDa laminin receptor was

14   demonstrated using a biotinylated derivative of the

15   peptide (Acetyl-C-[$S$-Acm]-VIGYSGDR-C-[$S$-Acm]-K-[$N^\epsilon$-

16   biotin]-amide) and a modification of the above laminin

17   attachment assay.

18

19   Briefly, 96-well plates were coated with 100 $\mu$l/well

20   streptavidin (5 $\mu$g/ml in carbonate buffer pH 9.6) and

21   following an overnight incubation at 37°C, wells were

22   washed with CFS (3 x 100 $\mu$l) and the plastic blocked

23   with casein (0.2% in CFS). The plates were then

24   incubated at room temperature for 45 min and washed

25   with CFS as previously detailed. Biotinylated mEGF$_{(33-42)}$

26   in CFS was then aliquoted into the wells (0.1 ml of 100

27   $\mu$M) and the plates incubated for 3 h at 37°C.

28

29   After a further block with 0.2% casein, the wells were

30   washed with CFS (3 x 100 $\mu$l aliquots). Plates were kept

31   at 4°C and used within 2 h.

32

33   Cells were prepared as above and pre-incubated for 1 h

34   at 37°C with serial dilutions of anti-laminin receptor

35   polyclonal (see below) or anti-EGF (R1) receptor

1    monoclonal antibodies. Subsequent procedures were as

2    detailed for the laminin attachment assay.

3

4    Laminin receptor binding determinations

5

6    a. Radiolabelling of laminin

7

8    $^{125}$I-laminin was prepared using $^{125}$I-labelled sodium

9    iodide (Amersham, UK) and immobilised chloramine-T

10   (Iodobeads; Pierce, Illinois). Prior to use, the beads

11   were washed with 500 $\mu$l phosphate buffer (pH 6.5) to

12   remove excess reagent from the support. These were then

13   allowed to air dry and individual beads added to a

14   solution of carrier free Na$^{125}$I, diluted with iodination

15   buffer (phosphate buffer pH 7.4). The beads were

16   allowed to equilibrate for 5 min.

17

18   Laminin (10 $\mu$g in 10 $\mu$l) was then diluted into the

19   iodination buffer and the system incubated at 20°C for

20   15 min. The solution was then removed from the reaction

21   vessel and excess Na$^{125}$I and unincorporated $^{125}$I$_2$

22   separated from the iodinated protein by gel filtration

23   on a GF-5 exclusion column (Pierce, Illinois).

24   Iodinated laminin fractions were recovered at a

25   specific activity of approximately 1.2 mCi/mg protein

26   (864 Ci/mmol).

27

28   b. Competition binding estimation

29

30   Near confluent cultures of T47-D or SK HEP-1 cells were

31   removed from monolayer with 0.02% EGTA and passed

32   through a G-25 syringe needle to produce single cell

33   suspensions. Aliquots of each cell type (10$^6$ cells/ml)

34   were dispensed into separate Ependorf tubes (1 ml each)

35   and pelleted. The cells were then resuspended in 1 ml

20

1   ice-cold RPMI (SK HEP-1) or DMEM (T47-D) containing
2   0.1% BSA and either laminin or synthetic peptide at the
3   concentrations indicated. Iodinated laminin was then
4   added to each cell suspension to give a final $^{125}$I-
5   laminin concentration of 0.1 nM (approximately 50,000
6   cpm). These mixtures were incubated overnight at 4°C.
7
8   The tubes were then microfuged at 10,000 g and the
9   supernatant removed. After washing the pellet with 500
10  μl CFS, the remaining radioactivity was determined
11  using a gamma radiation counter. Non-specific binding
12  was determined by incubating cells with a 1000-fold
13  molar excess of unlabelled laminin. All estimations
14  were carried out in triplicate.
15
16  $IC_{50}$ (concentration of unlabelled peptide required to
17  produce 50% inhibition of radioligand binding) and $EC_{50}$
18  (effective concentration for 50% inhibition of cell
19  attachment) values were calculated using the Grafit
20  curve-fitting programme (Erithacus Software, London,
21  UK).
22
23  **Migration assays**
24
25  The method used was basically as described by Albrecht-
26  Buehler (1977). Briefly, coverslips (22 x 22 mm) were
27  treated in 5% detergent (7X; ICN Biomedicals) and
28  washed in alcohol to remove grease. After drying, they
29  were immersed in gelatin solution (Sigma, 300 Bloom;
30  0.5 g in 300 ml distilled $H_2O$) for 10 min. The
31  coverslips were then dried by placing in a 70°C oven
32  for 45 min.
33
34  Colloidal gold suspension was prepared by adding 11 ml
35  distilled $H_2O$ and 6 ml $Na_2CO_3$ (36.5 mM) to 1.8 ml $AuHCl_4$

1    (14.5 mM). The mixture was heated to 95°C at which
2    point 1.8 ml of freshly prepared 0.1% formaldehyde
3    solution was added; the temperature was maintained at
4    95°C. A suspension of colloidal gold was formed which
5    was brown to absorbed light and blue to transmitted
6    light.
7
8    The gold suspension, was then added to petri dishes
9    containing individual coverslips and the plates
10   incubated at 37°C for 45 min. After washing with CFS (3
11   x 4 ml) to remove unattached gold particles, the
12   coverslips were transferred to 6-well cluster dishes
13   and UV sterilised.
14
15   Endothelial cells (SK HEP-1 and BRCE) in culture media
16   (0.3 ml) were seeded onto the coverslips at an
17   approximate density of $5 \times 10^3$ cells per well. The cells
18   were allowed to plate down for 2 h at 37°C after which
19   the treatments were added. Assay systems were
20   maintained for a further 18 h after which the cells
21   were fixed using 3% gluteraldehyde in cacodylate buffer
22   (pH 7.2).
23
24   The assays were examined using a Leica DM1RB phase
25   contrast microscope and Q500MC image analysis system
26   incorporating a JVC TK-1280E colour camera (Leica,
27   Milton Keynes, UK). The track images of at least 30
28   cells were video-captured and the area (representing
29   migration response) determined for each. Statistical
30   analysis of these areas was then carried out using
31   Macintosh Instat software to perform both Kruskal-
32   Wallis analysis of variance and Mann-Whitney $U$-tests in
33   order to compare the treatment groups with controls.
34
35   RESULTS

22

1    Proliferative response

2

3    All peptides were examined for their ability to
4    influence the growth of T47-D and SK-Hep 1 cell lines.
5    At concentrations of peptide up to 100$\mu$M, no
6    significant effects were observed in either cell line.

7

8    **Mechanism of action**

9

10    It had shown previously that mEGF$_{(33-42)}$ could inhibit the
11    EGF-stimulated angiogenic response in the early chick
12    as well as blocking the basal and EGF-stimulated
13    motility of primary and established endothelial cells.

14

15    During the present study it is shown that mEGF$_{(33-42)}$ also
16    inhibits the angiogenic effects of laminin (Nelson et
17    al 1995). Furthermore, it is demonstrated that the
18    anti-angiogenic effects of mEGF$_{(33-42)}$ are mediated solely
19    through the high affinity 67 kDa laminin receptor (67-
20    LR) and not through the EGF receptor.

21

22    The study also confirms that mEGF$_{(33-42)}$, Lam.$\beta$-1$_{(925-933)}$ and
23    laminin are equipotent in $^{125}$I-laminin displacement
24    receptor assays, and that both of the small peptidal
25    ligands have similar potencies in specific laminin cell
26    attachment assays.

27

28    In addition, it is shown that the commonly used chick
29    angiogenesis models are not appropriate to the study of
30    laminin mediated human angiogenesis: although it is
31    confirmed that Lam.$\beta$-1$_{(925-933)}$ acts as a partial laminin
32    antagonist in chick, it was found to be a pure agonist
33    in mammalian cell lines. This is a highly significant
34    point given that pharmaceutical companies (such as
35    Angiotech, Vancouver, BC) are using the chick CAM assay

1  as the sole screening method for the discovery of anti-

2  angiogenic lead compounds. This may be inappropriate

3  for use in human disease.

4

5  This study is the first to show that the YIGSR-receptor

6  is, in fact, the 67 kda high affinity laminin receptor

7  (67-LR). In collaboration with Professor Archer's team

8  at the Department of Ophthalmology, Royal Victoria

9  Hospital, Belfast, it has been determined that the 67-

10  LR is preferentially expressed in new vessels during

11  oxygen-induced retinopathy in neonatal mice.

12

13  **Peptide antagonist development**

14

15  The $N$-terminus of Lam.$\beta$-1$_{(925-933)}$ is not necessary for

16  receptor recognition and the agonist activity of YIGSR

17  peptide (Ostheimer et al 1992, Kawasaki et al 1994).

18

19  However, alanine scanning of the starting peptide

20  (mEGF$_{(33-42)}$) indicated that residues at positions 1, 2,

21  3, and 6 (peptidesVI, VII, VIII and X respectively),

22  are essential for receptor mediated activities as

23  determined by $^{125}$I-laminin displacement and cell

24  attachment to laminin through the 67-LR. Substitution

25  of these individual residues by alanine leads to a

26  dramatic decrease in receptor affinity observed as an

27  increased IC$_{50}$ (Table 2) and a parallel decrease in

28  their ability to block adhesion to laminin (increased

29  EC$_{50}$; Table 2). Characterisation of these analogues with

30  regard to effects on motility, largely confirmed these

31  findings although there was one exception; peptide

32  VIII. Results from the migration assay identified this

33  sequence (alanine for cysteine (P1)) as being a weak

34  laminin agonist despite there being a much reduced

35  response in the other two assays. It is suggested that

1    this peptide may influence laminin receptor mediated

2    migration through an alternative mechanism (Scott

3    1997).

4

5    Substitution at P10 (alanine for cysteine (peptide X)

6    retains both receptor binding and adhesion displacing

7    activities but has the effect of changing the

8    antagonistic parent into an agonist analogue. This

9    reflects the response the agonism of Lam.$\beta$-1$_{(925-933)}$,

10   which also lacks the C-terminal cysteine, and suggests

11   that this cysteine is not essential for receptor

12   recognition, but is required for antagonism of mEGF$_{(33-}$

13   $_{42)}$.

14

15   Studies have reported that the positive charge offered

16   by arginine (P9) is essential for the biological

17   activity of Lam.$\beta$-1$_{(925-933)}$ (McKelvey et al 1991, Kawasaki

18   et al 1994). Glutamate substitution for arginine

19   generates a negative charge at this position with

20   corresponding loss of biological activities (Kawasaki

21   et al 1994).

22

23   However, the substitution of arginine (P9) with

24   positively-charged lysine (McKelvey et al 1991) also

25   results in complete loss of ligand binding and

26   biological activities, suggesting that the mere

27   presence of a positive charge at this position is, in

28   itself, insufficient for receptor recognition. This

29   modelling studies suggest that H-bonding of the

30   guanidino group of the arginyl residue to the aromatic

31   sidechain of the tyrosyl residue (P5) in the consensus

32   sequence GYXGXR presents an acceptable motif for 67-LR

33   activation by both mEGF$_{(33-42)}$ and Lam.$\beta$-1$_{(925-933)}$.

34

35   Substitution of tyrosine (P5) with a conformationally

25

1    restricted mimetic (tetrahydroisoquinoline-3-carboxylic
2    acid; Tic-OH) in peptide V converted the antagonist
3    mEGF$_{(33-42)}$ into an agonist. This residue substitution
4    generates a predicted conformation unlikely to be able
5    to form H-bonds. Although both receptor binding and
6    adhesion responses were retained in this peptide the
7    loss of antagonism would suggest that H-bonding between
8    tyrosine (P5) and the arginine (P9) is important for
9    these antagonist activities.

10

11   Modelling studies suggested that citrulline (an
12   uncharged arginine mimetic) would also be capable of
13   forming this H-bonded motif.

14

15   It was found that replacement of arginine (P9) with
16   citrulline (peptide IV) increased both receptor binding
17   and inhibition of attachment to laminin substrata
18   whilst retaining antagonist migratory response (Table
19   2), reinforcing the observation that it is not the
20   positive charge that is required rather than an active
21   conformation generated by hydrogen bonding. These
22   findings thus identify H-bonding between P5 and P9 as
23   being more important than the charge at the P9 arginine
24   in determining antagonist activity.

25

26   Subsequent strategies involved the substitution of
27   variant residues in the antagonistic mEGF$_{(33-42)}$ with
28   those present in the agonistic Lam.$\beta$-1$_{(925-933)}$ sequence
29   (peptides I-III), in an effort to identify key amino
30   acids in the C-terminal regions (P5-10) of the two
31   ligands responsible for their contrasting
32   bioactivities.

33

34   Substitution of isoleucine (P6) for serine (peptide I)
35   resulted in both reduced receptor affinity and potency

1   in displacement of cell adhesion to laminin. However,
2   this analogue retained weak antagonist activities in
3   the motility assay. It is therefore of interest that
4   studies on the YIGSR sequence indicate that residue
5   substitution, at the position taken by isoleucine in
6   the pentapeptide, are well tolerated and may increase
7   potency (Kawasaki et al 1994).
8
9   Replacement of aspartate (P8) with serine (peptide II)
10  resulted in a complete loss of biological function. as
11  did peptide III encompassing both the former
12  (isoleucine (P6) for serine) and latter (serine (P8)
13  for aspartate) substitutions. Since this $mEGF_{(33-42)}$
14  analogue sequence (peptide II) encompasses the active
15  YIGSR amino acid sequence agonist, it is suggested that
16  this loss of activity may be attributed to the valine
17  (P2) and isoleucine (P3) residues in the $N$-terminal
18  half of $mEGF_{(33-42)}$. Alternatively, addition of a $C$-
19  terminal cysteine to the YIGSR sequence is known to
20  reduce potency (Kawasaki et al 1994). Additional
21  peptides incorporating the valine (P2) and isoleucine
22  (P3) substitutions are currently under investigation.
23
24  The determination of the minimum core peptide structure
25  is ongoing and involves similar characterisation
26  studies on a number of sequences truncated at the $C$-
27  terminal.
28
29  These studies have thus identified an important
30  antagonist of 67-LR mediated activities in peptide IV.
31  The sequence, (AcC(Acm)-VIGYSGD-[Cit]-C-(Acm)-$NH_2$.), may
32  provide an important template for anti-angiogenic drugs
33  in that it is resistant to cleavage by trypsin-like
34  proteases and has been identified as being more potent
35  than $mEGF_{(33-42)}$ in screening procedures.

1    <u>Advantages</u>

2

3    The advantages of the invention, and the ways in which

4    disadvantages of previously known arrangements are

5    overcome include:

6

7    1.    Unlike the native 67LR ligand (laminin $\beta-1_{925-933}$),

8          which is angiogenic in human models, the $mEGF_{33-42}$-

9          derived agents are anti-angiogenic in human

10         models.

11

12   2.    $mEGF_{33-42}$ has the advantage of inhibiting both

13         laminin- and EGF-stimulated angiogenesis.

14

15   3.    $mEGF_{33-42}$ prevents tumour cell attachment to

16         basement membranes.

17

18

1

422 Rec'd PCT/PTO 2 0 OCT 2000

1   **CLAIMS**

2

3   1   A peptide factor derived from amino acid residues 33
4       to 42 of murine epidermal growth factor peptide or a
5       synthetic equivalent thereof wherein the peptide
6       factor is modified to protect it from proteolytic
7       degradation and the peptide binds to laminin
8       receptors wherein the modifications consist of at
9       least one modification chosen from the group
10      comprising; substitution of tyrosine by tyrosine
11      analogues and substitution of arginine by arginine
12      analogues,

13

14  2   A peptide as claimed in Claim 1 wherein the
15      peptide is further modified by at least one
16      modification chosen from the group comprising
17      capping the N terminal of the peptide, capping
18      the C terminal of the peptide and capping thiol
19      groups of cysteines.

20

21  3   A peptide as claimed in claims 1 or 2 wherein
22      tyrosine is substituted by Tic-OH.

23

24  4   A peptide as claimed in claims 1, 2 or 3 wherein
25      arginine is substituted by Citrulline.

26

27  5   Use of a peptide factor as claimed in any of the
28      preceding claims in the preparation of a medicament
29      to bind laminin receptors as an antagonist.

30

31  6   Use of a peptide factor as claimed in any of the
32      preceding claims in the preparation of a medicament
33      to bind laminin receptors as an agonist.

34

35  7   Use as claimed in claim 6 in the preparation of a
36      medicament for healing endothelial cell wounding.

AMENDED SHEET

2

1  8    Use as claimed in claim 8 or 9 for the treatment of
2       retinopathy of immaturity.
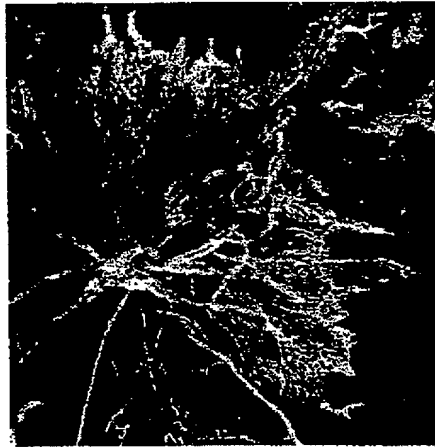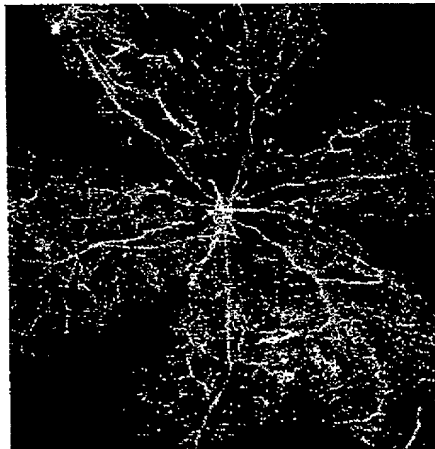
09/673785

Fig 1a



Fig 1b

| | Docket No. |
|---|---|
| CONFIDENTIAL | 41934/23838 |

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**PEPTIDE FRAGMENTS OF MURINE EPIDERMAL GROWTH FACTOR AS LAMININ RECEPTOR TARGETS**

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on   21.04.99                          as United States Application No. or PCT International

Application Number   PCT/GB99/01211

and was amended on   16.06.2000

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)                                        Priority Not Claimed

| 9808407.2 | GB | 22.04.98 | ☐ |
|---|---|---|---|
| (Number) | (Country) | (Day/Month/Year Filed) | |
| | | | ☐ |
| (Number) | (Country) | (Day/Month/Year Filed) | |
| | | | ☐ |
| (Number) | (Country) | (Day/Month/Year Filed) | |

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

| _____ | _____ |
| (Application Serial No.) | (Filing Date) |

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| _____ | _____ |
| (Application Serial No.) | (Filing Date) |

| _____ | _____ |
| (Application Serial No.) | (Filing Date) |

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

| PCT/GB99/01211 | 21.04.99 | PENDING |
| (Application Serial No.) | (Filing Date) | (Status) (patented, pending, abandoned) |
| (Application Serial No.) | (Filing Date) | (Status) (patented, pending, abandoned) |
| (Application Serial No.) | (Filing Date) | (Status) (patented, pending, abandoned) |

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.   *(list name and registration number)*

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